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(54) Title: DETECTION METHOD FOR C-RAF-1 GENES

(57) Abstract

The present invention relates to: (1) a method of identifying an individual at an increased risk for developing cancer, (2) a method for determining a prognosis in patients afflicted with cancer, and (3) a method for determining the proper course of treatment for a patient afflicted with cancer; comprising: amplifying a region of the c-raf-1 gene.

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# DETECTION METHOD FOR C-RAF-1 GENES

<u>Field of the Invention</u>

The present invention relates to (1) a method of identifying an individual at an increased risk for developing cancer, (2) a method for determining a prognosis of patients afflicted with cancer, and (3) a method for determining the proper course of treatment for a patient afflicted with cancer.

<u>Background Information</u>

Lung cancer claims more lives in the United States than any other neoplasm (R.S. Finley, <u>Am. Pharm.</u> NS29, 39 (1989)), and of the various forms lung adenocarcinomas have one of the worst prognoses (T.P. Miller, <u>Semin. Oncol.</u> 17, 11 (1990)). The incidence of adenocarcinoma of the lung (ACL) in the United States is also quickly rising (I. Linnoila, Hematol. <u>Oncol. North. Am.</u> 4, 1027 (1990); J.B. Sorensen, H.H. Hansen, <u>Cancer Surviv.</u> 8, 671 (1989)). In order to gain insight into this complex and deadly disease, a model system for its study has been developed. For such a model to provide clinically relevant data several criteria must be met. The tumors produced should be histologically equivalent to their human counterparts, tumor induction must be reliable and reproducible, and the numbers generated must be great enough to provide statistical significance. To satisfy these conditions a system has been created which uses two inbred mouse strains (NFS/n and AKR) along with

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transplacental exposure to the potent carcinogen N-ethyl-
N-nitrosourea (ENU) and promotion with the antioxidant
butylated hydroxytoluene (BHT). The resulting tumors were
examined for altered expression or structural mutations of
5 genes implicated in lung tumor development such as ras,
myc, and raf oncogenes (C.D. Little et al., Nature 306,
194 (1983); P.E. Kiefer et al., Cancer Res., 47, 6236
(1987); E. Santos et al., Science 223, 661 (1984); S.
Rodenhuis, N. Engl. J. Med. 317, 929 (1987); M. Barbacid,
10 Eur. J. Clin. Invest. 20, 225 (1990); U.R. Rapp et al., J.
Int. Assoc. for the Study of Lung Cancer 4, 162 (1988);
M.J. Birrer et al., Ann. Rev. Med. 40, 305 (1989); G.
Sithanandam et al., Oncogene 4, 451 (1989)).

15            raf proto-oncogenes are evolutionarily highly
conserved genes encoding cytoplasmic serine/threonine
specific kinases, which function in mitogen signal
transduction (reviewed in U.R. Rapp et al., The Oncogene
Handbook, T. Curran et al., Eds. (Elsevier Science
20 Publishers, The Netherlands, 1988), pp. 115-154; U.R.
Rapp, Oncogene 6, 495 (1991)). The three known active
members in the raf family encode phosphoproteins of
similar size (72/74 kD for Raf-1; 68 kD for A-Raf-1, and
74 kD for B-Raf (U.R. Rapp et al., in Retroviruses and
25 Human Pathology, R. Gallo et al., Eds. (Humana Press,
Clifton, New Jersey 1985), pp. 449-472; T.W. Beck et al.,
Nucleic Acids Res. 15, 595 (1987); G. Sithanandam et al.,
Oncogene 5, 1775 (1990))). Raf-1 was first identified as
the cellular homologue of v-raf (H.W. Jansen et al.,
30 Nature 307, 218 (1984)), the transforming gene of 3611 MSV
(U.R. Rapp et al., J. Virol. 45, 914 (1983); U.R. Rapp et
al., Proc. Natl. Acad. Sci. USA 80, 4218 (1983)). Amino
acid comparisons of raf family genes shows three conserved
regions [CR1, CR2, CR3] (T.W. Beck et al., Nucleic Acids
35 Res. 15, 595 (1987)); CR1 is a regulatory region
surrounding a Cys finger consensus sequence, CR2 is a
serine/threonine rich region, and CR3 represents the

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kinase domain. Raf-1 has been mapped to chromosome 3p25
in humans (S.J. O'Brien et al., Science 223, 71 (1984)),
and this region has been found to be frequently altered in
small cell lung carcinoma (SCLC) (J. Whang-Peng et al.,

5    Cancer Genet. Cytogenet. 6, 119 (1982); J.M. Ibson et al.,
J. Cell. Biochem. 33, 267 (1987)), familial renal cell
carcinoma (A.J. Cohen et al., N. Engl. J. Med. 301, 592
(1979); G. Kovacs et al., Int. J. Cancer 40, 171 (1987)),
mixed parotid gland tumors (J. Mark et al., Hereditas 96,

10   141 (1982)), and ovarian cancer (K. Tanaka et al., Cancer
Genet. Cytogenet. 43, 1 (1989)).


          Raf genes are differentially expressed in
various tissues (S.M. Storm et al., Oncogene 5, 345

15   (1990)). c-raf-1 has been found to be expressed
ubiquitously, though absolute levels vary between tissues.
A-raf-1 is present predominantly in the urogenital
tissues, whereas B-Raf is most abundant in cerebrum and
testis. The ubiquitous c-Raf-1 kinase is regulated by

20   tyrosine and serine phosphorylations that result from
activated growth factor receptor kinases (D.K. Morrison et
al., Cell 58, 648 (1989); D.K. Morrison et al., Proc.
Natl. Acad. Sci. USA 85, 8855 (1989); K.S. Kovacina et
al., J. Biol. Chem. 265, 12115 (1990); P.J. Blackshear et

25   al., J. Biol. Chem. 265, 12131 (1990); M.P. Carroll et
al., J. Biol. Chem. 265, 19812 (1990); J.N. Siegel et al.,
J. Biol. Chem. 265, 18472 (1990); B.C. Turner et al.,
Proc. Natl. Acad. Sci. USA 88, 1227 (1991); M. Baccarini
et al., EMBO J. 9, 3649 (1990); H. App et al., Mol. Cell.

30   Biol. 11, 913 (1991)). Raf-1 operates downstream of Ras
in mitogen signal transduction as judged by experiments
using antibody microinjection (M.R. Smith et al., Nature
320, 540 (1986)), c-raf-1 antisense expression constructs
(W. Kolch et al., Nature 349, 426 (1991)), dominant

35   negative mutants (W. Kolch et al., Nature 349, 426
(1991)), and Raf revertant cells. Studies with NIH3T3
cells and brain tissue demonstrated that mitogen treatment

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induces Raf-1 kinase activity and causes a transitory relocation of the active enzyme from the cytoplasm to the nucleus and perinuclear area (Z. Olàh et al., Exp. Brain. Res. (in press); U.R. Rapp et al., in Cold Spring Harbor

5   Symposia on Quantitative Biology, Vol. LIII, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1988) pp. 173-184).

Raf-1 coupling has been examined in more than a

10  dozen receptor systems and all strong mitogens stimulated Raf-1 kinase activity (U.R. Rapp, Oncogene 6, 495 (1991); D.K. Morrison et al., Cell 58, 648 (1989); D.K. Morrison et al., Proc. Natl. Acad. Sci. USA 85, 8855 (1989); K.S. Kovacina et al., J. Biol. Chem. 265, 12115 (1990); P.J.

15  Blackshear et al., J. Biol. Chem. 265, 12131 (1990); M.P. Carroll et al., J. Biol. Chem. 265, 19812 (1990); J.N. Siegel et al., J. Biol. Chem. 265, 18472 (1990); B.C. Turner et al., Proc. Natl. Acad. Sci. USA 88, 1227 (1991); M. Baccarini et al., EMBO J. 9, 3649 (1990); H. App et

20  al., Mol. Cell. Biol. 11, 913 (1991)), and this stimulation correlated with an increase in Raf-1 phosphorylation leading to a shift in apparent molecular weight.

25                    SUMMARY OF THE INVENTION

It is an object of this invention to provide a method of identifying an individual at an increased risk for developing cancer.

30

It is another object of this invention to provide a method for determining a prognosis in patients afflicted with cancer.

35           It is a further object of this invention to provide a method for determining the proper course of treatment for a patient afflicted with cancer.

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Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates
5   to a method of identifying an individual at an increased risk for developing cancer, comprising:
     amplifying a region of the c-raf-1 gene;
     analyzing products of the amplification for evidence of mutation; and
10       classifying an individual having one or more mutations in the region as having an increased risk for developing cancer.

In another embodiment, the present invention
15  relates to a method for determining a prognosis in patients afflicted with cancer, comprising:
     amplifying a region of the c-raf-1 gene;
     analyzing products of the amplification for evidence of mutation; and
20       classifying patients having no mutation in said region as being less likely to suffer disease relapse or having an increased chance of survival than those patients having one or more mutations in said region.

25       In a further embodiment, the present invention relates to a method for determining the proper course of treatment for a patient afflicted with cancer, comprising:
     amplifying a region of the c-raf-1 gene;
     analyzing products of said amplification for evidence
30  of mutation;
     identifying a patient having at least one mutation in said region, which patient may require treatment proper for patients having a lesser chance of survival or decreased time to relapse; and
35       identifying a patient lacking mutations in said region, which patients may require treatment proper for

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patients having a greater chance of survival or being less likely to suffer disease relapse.

BRIEF DESCRIPTION OF THE DRAWINGS

5            Figure 1. Effect of BHT promotion on ENU tumorigenesis in NFS/n x AKR mice. The X-axis represents percent tumor induced mortality within each group, and the Y-axis reflects age in weeks. All animals were exposed to
10   ENU transplacentally at a dose of 0.5mM/Kg of mother's body weight on day 16 of gestation (presence of vaginal plug was scored as day one). At two weeks of age mice were weaned into two separate groups and separated by sex. Both groups were housed in identical cages and supplied
15   with food (Purina Lab Chow) and water ad libitum. Beginning at three weeks of age, group 2A (O) was given weekly intraperitoneal (i.p.) injection of corn oil (0.1 ml), and group 2B (◊) received weekly i.p. injections of BHT (20 mg/Kg of body weight) dissolved in corn oil.
20   Administration of BHT reduces the mean age of mortality from approximately 20 weeks to 13, and decreases the initial age of mortality. These curves are significantly different (p≤ 0.001) as judged by a 2-tailed Cox test. In both groups the rate of tumorigenesis was identical for
25   males and females.

            Figure 2. Northern blot analysis of proto-oncogene expression levels in ENU induced tumors.

30            Figure 3. Diagnostic digestion of PCR amplified Ki-ras genes from ENU induced tumors. Genomic DNA was isolated from a cesium chloride gradient during RNA preparations. In each case 10 ng was amplified via PCR (95°C, 5 min. followed by 35 cycles of 95°C, 1 min. →
35   55°C, 1 min. → 72°, 1 min.) with 2 units of Taq I polymerase. The primers used (K1; 5'-AACTTGTGGTGGTTGGACCT-3'→ (SEQ ID NO:6) and K2; <= 3'-

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GTCTTAGTGAAACACCTACT-5' (SEQ ID NO:7)) generate a 79 b.p.
product. The primer K1 ends at codon 12 and contains a
mismatch from normal mouse (Ki-ras sequence its 18th
nucleotide (G → C) creating a BstNI site (CCTGG) in
5    conjunction with a normal codon 12 (GGT). Digestion of
amplified product from a normal allele with BstNI produces
two products of 19 and 60 b.p., whereas a mutation in one
of the first two positions of codon 12 will eliminate the
BstNI site. The presence of two normal alleles results in
10   all of the product being cleaved and the presence of one
mutant and one normal allele will result in only half of
the product cut. In the three panels each sample was run
in duplicate, uncut and cut with BstNI. F1 is DNA from an
untreated NFS/n X AKR F1 mouse, and MCA5 is a murine cell
15   line known to harbor a mutant K-ras codon 12 allele. One
lymphoma (24Ly) and one cell line (117; derived from a
lung adenocarcinoma) display a mutated Ki-ras codon 12
allele; however, 24Ly was a passaged tumor and examination
of the original tumor showed two normal alleles indicating
20   that this mutation was acquired during passaging.


Figure 4. c-raf-1 RNAse protection analysis of
ENU induced tumors. The probe used was a 32P labeled
antisense transcript from the 3' non-coding region of a
25   mouse c-raf-1 cDNA to the 3' most StuI site.
Hybridization of this probe with normal RNA results in a
protected fragment of 1.2kb covering the region encoding
the Raf-1 kinase domain. One µg of poly(A)+ RNA from each
tumor and 5 µg of F1 RNA (in order to get comparable
30   signals) was hybridized for 12 hours at 52°C with 200,000
cpm of $^{32}$P labeled mouse c-raf antisense transcript.
Hybrids were then digested for 30 minutes with 25 µg RNAse
A and 33 units of RNAse T1 at room temperature. Digested
hybrids were then incubated with 50 µg of proteinase K,
35   phenol/chloroform extracted, ethanol precipitated, and
resuspended in loading dye containing 80% formamide.
Samples were then run on 6% polyacrylamide denaturing

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sequencing gels at 65 watts. Gels were vacuum dried at 80 degrees C and exposed to X-ray film. Probe is undigested probe alone; tRNA is probe hybridized to non-specific RNA; v-raf is probe hybridized to RNA from a v-raf transformed
5   cell line and the bands detected represent single base mismatches between murine c-raf and v-raf; NFS/AKR F1 is probe hybridized with RNA from a normal (untreated) mouse; 24 LY is probe hybridized with RNA from a lymphoma; and the remaining lanes are probe hybridized with RNA isolated
10  from lung tumors.

         Figure 5. Schematic of Raf-1 protein showing sites of ENU induced mutations. CR1, CR2, and CR3 represent conserved regions 1, 2 and 3. cDNAs were made
15  from tumor derived poly(A)+ RNA using MoMuLV reverse transcriptase. Primers (MR1 sequence and MR2 sequence) encompassing a 435 base pair region c-raf were then used to amplify this region via PCR. The amplification mixture was then run on 1.7% agarose gels and the 435 bp product
20  isolated. This isolated fragment was then treated with T4 polymerase and cloned into the HincII site of M13mp18 for sequencing. Another set of primers (EMR1 sequence and EMR2 sequence) was designed containing EcoRI sites at the termini and used to amplify a 609 base pair region
25  (encompassing the original 435 base pair region). Isolated products from these reactions were then digested with EcoRI and cloned into the EcoRI site of KS. Sequencing reactions were carried out using the Sequenase kit (USB) according to the recommended protocols for
30  single and double stranded sequencing. Sequencing reactions were run on 6% polyacrylamide denaturing gels at 65 watts. Gels were vacuum dried at 80 degrees C and exposed to X-ray film. In each case a normal allele was also sequenced along with the mutant allele.

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Figure 6.  Schematic for Identifying c—raf—1
mutations.  Primers 1 and 2 are shown in SEQ ID NO:8 and
SEQ ID NO:9, respectively.

5               DETAILED DESCRIPTION OF THE INVENTION

        The present invention relates to methods that
involve amplifying a region of the c—raf—1 gene (the
sequence of a mouse c—raf—1 gene is shown in SEQ ID NO:10;
10  the nucleotide and corresponding amino acid sequence of a
human c—raf—1 gene is shown in SEQ ID NO:11 and SEQ ID
NO:12, respectively).

        In one embodiment, the present invention relates
15  to a method of identifying an individual at an increased
risk for developing cancer (preferably, lung cancer, T—
cell lymphomas, renal cell carcinoma, ovarian carcinoma,
and mixed parotid gland tumors) comprising: amplifying a
region (preferably by using the polymerase chain reaction
20  method(PCR) or by cloning techniques) of the c—raf—1 gene
of the individual (SEQ ID NO:11)(in one preferred
embodiment, the region encodes amino acids 514 to 535 of
SEQ ID NO:12); analyzing products of the amplification for
evidence of mutation (preferably by DNA sequencing of the
25  region) and classifying an individual having one or more
mutations in the region as having an increased risk for
developing cancer.  In one prefered embodiment, the region
encodes amino acids 500 to 550 of SEQ ID NO:12 or amino
acids 450 to 630 of SEQ ID NO:12.  In another prefered
30  embodiment, the PCR method employs a primer comprising the
sequence shown in SEQ ID NO:7 and a primer comprising the
sequence shown in  SEQ ID NO:8.  In another prefered
embodiment, the method comprises the steps shown in Figure
6.
35          In another embodiment, the present invention
relates to a method for determining a prognosis in a
patient afflicted with cancer (preferably, those cancers

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listed above). The method comprises: amplifying the region of the c-raf-1 gene as described above; analyzing products of the amplification for evidence of mutation as described above; and classifying a patient having no
5  mutation in the region as being less likely to suffer disease relapse or having an increased chance of survival than a patient having one or more mutations in the region.

In another embodiment, the present invention
10  relates to a method for determining the proper course of treatment for a patient afflicted with cancer (preferably, those cancers listed above), comprising: amplifying a region (described above) of the c-raf-1 gene as described above; analyzing products of the amplification for
15  evidence of mutation as described above; identifying a patient having at least one mutation in the region, which patient may require treatment proper for patients having a lesser chance of survival or decreased time to relapse; and identifying a patient lacking mutations in the region,
20  which patients may require treatment proper for patients having a greater chance of survival or being less likely to suffer disease relapse.

Administration of therapeutic agents (cytotoxic
25  or cytostatic) tailored to recognize the mutant Raf-1 protein but not normal Raf-1 could specifically target tumor cells for death of growth inhibition. Such agents could be comprised of cytotoxic T-cells, antibodies, and/or specifically designed chemical compounds.

30
The following Examples demonstrate consistent point mutations of the c-raf-1 proto-oncogene, within a small region of the kinase domain, in a mouse model for chemical tumor induction. This is the first demonstration
35  of point mutated raf genes in vivo, and the first isolation of activating in vivo point mutations in the kinase domain of a proto-oncogene. The tumors examined

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show a selective specificity for Raf-1 mutations as
another family of genes, the ras proto-oncogenes which are
frequently activated by point mutation in both animal and
human tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis.
5    142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299
(1991)), is not involved.


The present invention is described in further
detail in the following non-limiting examples.
10

EXAMPLES
The following protocols and experimental details
are referenced in the examples that follow:


15   RNA Isolation. Tumors were excised, a small portion
minced in PBS (phosphate buffered saline solution) for
passaging in nude mice, frozen immediately in a dry
ice/ethanol bath, and stored at -70° until RNA extraction.
Frozen tissues were minced on wet ice in a guanidine
20   thiocyanate buffer (4M guanidine thiocyanate 10mM EDTA, 2%
N-lauryl sarcosine, 2% beta-mercaptoethanol, 10mM Tris
(pH=7.6)), disrupted in a Dounce homogenizer, and
extracted three times with phenol: chloroform: isoamyl
alcohol (24:24:2). Supernatants were then transferred to
25   SW41 tubes, 100 µg of cesium chloride per ml was added to
the supernatant which was then underlayed with one half
saturated cesium chloride in 10mM EDTA (pH=7.0; index of
refraction 1.3995-1.4000), and centrifuged at 25,000 rpm
for 20 hours in a Sorvall SW-41TI rotor using a Beckman
30   model L5-50 ultracentrifuge. Supernatants were removed
and RNA pellets dissolved in 4 ml resuspension buffer (10
mM Tris-HCl pH=7.6, 5% beta-mercaptoethanol, 0.5% N-lauryl
sarcosine, 10 mM EDTA), extracted once with
phenol:chloroform:isoamyl alcohol, sodium acetate added to
35   0.12M and RNA precipatated with two volumes ethanol at -
20°C overnight. Precipitates were centrifuged at 9,000 rpm
in a Sorvall SS-34 rotor for 30 minutes, and pellets

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redissolved in RNA sample buffer (10 mM Tris pH=7.4, 1mM
EDTA, 0.05% sodium dodecyl sulfate) and concentrations
determined by absorbance at 260 nm.  Poly (A)⁺ RNA was
isolated by binding to oligo dT cellulose columns in high
5  salt (10mM Tris pH=7.4, 1mM EDTA, 0.05% SDS, 500mM NaCl),
and eluting with RNA sample buffer heated to 40°C.

Northern Blotting.  5 μg poly(A)⁺ RNA per lane was ethanol
precipitated, desiccated, resuspended in loading buffer
10  (20mM MOPS pH=6.8, 5mM sodium acetate, 1mM EDTA, 50%
formamide, 6% formaldehyde), heated at 65°C for 5 min.,
quick chilled on wet ice for 10 min., and electrophoresed
through a 0.7% agarose gel containing 2.2 M formaldehyde,
20mM MOPS [pH=6.8], 5mM sodium acetate, and 1mM EDTA.
15  Gels were then blotted overnight onto nitrocellulose
filters via capillary transfer in 20X SSC, filters were
washed in 3X SSC for 10 min. and baked at 80°C for 2
hours.

20  Hybridizations.  Filters were prehybridized at 42°C in 5X
SSC, 50% formamide, 20mM sodium phosphate pH=6.8, 200
μg/ml PVP-40, 200 μg/ml ficoll 400, 200 μg/ml bovine serum
albumin, and 200 μg/ml sonicated sheared salmon sperm DNA.
Blots were then hybridized with 500,000 cpm/ml of random
25  primed ³²P labeled probes overnight at 42°C in
prehybridization solution with 5% dextran sulfate.  Blots
were washed with agitation in 2X SSC, 0.1% SDS at room
temperature six times for 20 minutes each wash, then
washed once at 45°C in 0.1X SSC for 15 minutes.  Filters
30  were exposed to X-AR 5 film at −70°C.

## EXAMPLE 1
### Tumor Induction

35          NFS female mice were mated with AKR males and
pregnant females given a transplacental injection of 1-
ethyl-1-nitrosourea (ENU) at a dosage of 0.5 mM/Kg

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mother's body weight on day 16 of gestation, counting plug
date as day one.  ENU was chosen for tumor induction since
it is a very potent direct acting carcinogen capable of
modifying any base in vivo (Singer, B. et al., 1983.

5    Molecular Biology of Mutagens and Carcinogens, Plenum
Press, New York).  ENU alkylates all tissues with roughly
the same efficiency (E. Scherer et al., Cancer Lett. 46,
21 (1989)) and has a very short half life in vivo (E.M.
Faustman et al., Teratology 40, 199 (1989)) allowing

10   specific mutagenesis of tissues which are mitotically
active at a particular time.  NFS and AKR were chosen as
parental strains based on earlier studies which showed
them to be particularly susceptible to ling tumors
following ENU exposure (B.A. Diwan et al., Cancer Res. 34,

15   764 (1974); S.L. Kauffman, JNCI 57, 821 (1976)).  With
this procedure nearly 100% of the offspring develop lung
adenocarcinomas and approximately 70% develop, in
addition, T-cell lymphomas with a mean latency of
approximately 20 weeks.  In order to achieve more rapid

20   tumor development,weanling mice were treated with weekly
injections of a tumor promoter, the antioxidant butylated
hydroxytoluene or BHT (20mg/kg body weight dissolved in
corn oil).  BHT was used as it has been demonstrated to
cause lung lesions and hyperplasia when injected into mice

25   (A.A. Marino et al., Proc. Soc. Exp. Biol. Med. 140, 122
(1972); H. Witschi et al., Proc. Soc. Exp. Biol. Med.,
147, 690 (1974); N. Ito et al., CRC Crit. Rev. Toxicol.
15, 109 (1984)).  In the present system it nearly doubles
the rate at which tumors develop.  Figure 1 compares tumor

30   induced mortality with age of animals for those receiving
ENU alone, and those receiving ENU and promoted with BHT.
These curves demonstrate that when BHT is given the mean
age of tumor induced mortality decreases from
approximately 20 weeks to around 12, and there is also a

35   decrease in initial latency.  These curves are
significantly different with a confidence limit greater
than 99.99% using a 2-tailed Cox test.  In addition, BHT

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promotion, while increasing the rate at which tumors
develop, does not affect the tumor spectrum.

EXAMPLE 2

Oncogene Expression

5

        Northern blot analysis revealed elevated levels
of c-raf-1, as compared to normal tissue, in every tumor
examined (Figure 2), and Western blot analysis showed that
10  protein levels correlated with message levels (U.R. Rapp
et al., in Oncogenes and Cancer, S.A. Aaronson et al.,
Eds. (Tokyo/VNU Scientific Press, Tokyo, 1987) pp. 55-74).
In addition, in cell lines derived from primary tumors,
Raf-1 protein kinase activity was shown by immune-complex
15  kinase assays to be constitutive.  Further analysis of
other oncogenes revealed no consistent pattern of
expression except for ras and myc family genes.  In the
case of the myc family, one member (either c-, N-, or L-
myc) was overexpressed but never more than one.  For the
20  ras genes, at least one member (Ki-, Ha-, or N-ras), and
often more than one, was expressed at high levels when
compared with the normal tissue.  In addition all
oncogenes examined via Northern analysis exhibited full
length, normal sized transcripts.

25

        ras genes were considered likely candidates for
mutational activation since oncogenic forms of Ki-ras have
previously been observed in lung tumors (S. Rodenhuis et
al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et
30  al., Carcinogenesis 12, 299 (1991)) and ENU is a point
mutagen (Singer, B. et al., 1983. Molecular Biology of
Mutagens and Carcinogens, Plenum Press; New York).  A
systematic analysis of various ras codons known to be
involved in oncogenic activation was therefore performed .
35  Ha-, Ki-, and N- ras were examined at codons 12, 13, and
61 for potential mutations via RNAse protection assays
(R.M. Myers et al., Science 230, 1242 (1985); E. Winter et

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al., Proc. Natl. Acad. Sci. USA 82, 7575 (1985)), PCR
amplification followed by subsequent sequencing (F. Sanger
et al., J. Mol. Biol. 13, 373 (1975)), and PCR
amplification followed by diagnostic restriction digests
5   (W. Jiang et al., Oncogene 4, 923 (1989)).  PCR
amplification creating diagnostic enzyme sites is a very
efficient way of examining alleles for mutations at known
sites and involves designing a PCR primer whose 3' end
lies next to and produces a novel restriction site
10  encompassing the codon of interest.  Following
amplification, PCR products from normal alleles will
contain the new restriction site, while mutant alleles
will not.  Digestion of the product from tissue with two
normal alleles results in all product being cut; however,
15  if one allele contains a mutation, only half of the
product will be digested.  Figure 3 shows the results of
amplification and diagnostic digestion applied to Ki-ras
codon 12 in several tumors and cell lines.  The first
panel is from a set of lymphomas.  F1 is DNA from a normal
20  untreated mouse and both alleles are cut by BstN1,
indicating the presence of two normal alleles.  MCA5 is a
murine cell line known to contain a Ki-ras codon 12
mutation (L.F. Parada et al., Mol. Cell. Biol. 3, 2298
(1983)), and only the amplified normal allele is cleaved.
25  Of the five tumors shown in the second panel, one shows a
mutant Ki-ras allele.  The next panel shows some of the
lung tumors tested and none of them exhibit a mutant
allele, and the final panel shows tumor derived cell
lines.  The first three are from lymphomas and the last
30  three from lung adenocarcinomas.  One lung tumor line
(#117) has a Ki-ras 12 mutation that was not present in
the primary tumor but came up upon transplantation.  This
analysis has been performed with Ki, Ha and N-ras genes at
codons 12 and 61.  Of all the tumors and cell lines
35  examined by this method for mutations of the three ras
genes at codons 12 and 61, the two shown here were the
only ones detected.  Examination of codon 13 was done by

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PCR amplification of genomic DNA surrounding codon 13 followed by cloning into KS+ (Stratagene) and double stranded sequencing. Table I summarizes the ras mutation data. The most notable point from this table is the

5 conspicuous lack of ras mutations in these tumors. In fact the number of ras mutations is much lower than would be expected for a sampling of spontaneous tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299 (1991); J.L. Bos,

10 Cancer Res. 49, 4682 (1989)). Having eliminated ras genes as playing a primary role in the genesis of these ENU induced tumors, c-raf-1 was investigated for possible small or point mutations.

15 TABLE I
Tumors and Cell Lines Positive for ras Mutations

| | Codon 12 | | Codon 13 | | Codon 61 | |
|---|---|---|---|---|---|---|
| | Tumors | Cell Lines | Tumors | Cell Lines | Tumors | Cell Lines |
| Ha-ras | 0/10 | 0/6 | 0/6 | 0/2 | 0/10 | 0/6 |
| Ki/ras | 1*/10 | 1/6 | 0/6 | 0/2 | 0/6 | 0/2 |
| N-ras | 0/10 | 0/6 | 0/6 | 0/2 | 0/10 | 0/6 |

* This was a second passage tumor in which the original
25 tumor did not contain a Ki-ras mutation.

Table I: Summary of mutation analysis for Ha-, Ki-, and N-ras at codons 12, 13, and 61. Each box displays the number of mutations detected, over the number of tumors
30 and tumor derived cell lines examined via RNAse protection, sequencing or diagnostic digestion, for each of the nine codons.

EXAMPLE 3

35 Mutations in Raf-1

Since no point mutations had been described for raf genes in vivo, as had been for the ras genes (E.

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Santos et al., Science 223, 661 (1984); S. Rodenhuis, N.
Engl. J. Med. 317, 929 (1987); M. Barbacid, Eur. J.
Clin. Invest. 20, 225 (1990); F. Sanger et al., J. Mol.
Biol. 13, 373 (1975)), point mutations were screened for
using RNAse protection assays (R.M. Myers et al.,
Science 230, 1242 (1985); E. Winter et al., Proc. Natl.
Acad. Sci. USA 82, 7575 (1985)).  Figure 4 shows a
typical protection assay using a c-raf-1 probe.  In this
experiment the probe used covered the 3' end of raf-1
from the 3' most StuI site to the end of the coding
sequence.  The first lane is a marker (pBR322 digested
with HaeIII), the second shows the probe alone
undigested, the third lane shows the probe hybridized to
unrelated RNA in this case tRNA, the fourth lane shows
hybridization with v-raf transformed cells and the lower
bands represent cleavage at points where the mouse c-
raf-1 gene differs from v-raf.  The fifth lane shows
hybridization with RNA isolated from a normal lung of an
untreated F1 mouse, the next lanes are RNA isolated from
several tumors.  In the case of the normal RNA, only
one, fully protected, band is detected while in the case
of the tumors two major bands are seen after digestion.
20 out of 20 tumors analyzed in this fashion showed this
extra band.  These data demonstrate the following major
points:  1) there is a tumor specific alteration in c-
raf-1 that results in a region of non-homology
recognizable by either RNAse A or T1; 2) The alterations
are confined to the same region of one allele as two
bands of equal size are present in the tumor lanes, and;
3) both alleles were expressed at comparable levels as
both bands are of approximately equal intensity.  In the
assay shown 5 µg of poly(A)+ RNA was hybridized from
normal tissue, and 1 µg was used from the tumors.  This
was necessary to get signals that could be compared on
the same gel due to the overexpression of c-raf-1 in the
tumors.  By running these assays with various markers it
was possible to estimate the approximate site of the

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alteration(s) to be in the vicinity of the exon 14/exon
15 junction. In order to define the precise genetic
alteration or alterations, PCR primers were designed
which would generate a 600 bp fragment encompassing this

5 region. cDNAs from tumor derived RNA were then
amplified and cloned into KS+ (Stratagene) for double
stranded sequencing. The sequencing results from
several tumors are shown in Figure 5. The top portion
of Figure 5 presents a cartoon of the mouse Raf-1

10 protein. There are three conserved regions CR1, CR2 and
CR3 with CR3 representing the kinase domain. The probe
used in the RNAse protection assays covers the indicated
area, and the PCR primers amplified the bracketed
region. Sequencing through this area revealed a variety

15 of mutations just downstream of the APE site. These
mutants are shown in an expanded version at the bottom
of Figure 5 (See also SEQ ID NO:1 for normal mouse
sequence and SEQ ID NO:2, 3, 4, and 5 for mutant
sequences). These mutants were isolated from four

20 separate tumors, and in each case a normal allele (SEQ
ID NO:1) was also sequenced. Repeating the cDNA
synthesis, PCR amplification, cloning and sequencing
gives the same sequence and normal tissue shows no
mutations demonstrating that these alterations are not

25 artifactual. Sequence covering the amplified region has
been examined and it is interesting that all of these
changes occur within a very small region of the raf
protein. In fact the region where these mutations occur
overlaps an epitope shared by monoclonal antibodies

30 generated against raf (W. Kolch et al., Oncogene 5, 713
(1990)), and computer modeling of the protein shows this
to be a hydrophilic domain, the structure of which is
predicted to be altered by these mutations. This
indicates a biologically important region for the

35 molecule and indeed the first of these mutation tested
in NIH3T3 cell assays, after cloning into a retroviral
expression vector (E1-neo, (G. Heidecker et al., Mol.

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<u>Cell. Biol.</u> 10, 2503 (1990))), was found to be weakly
transforming when driven by a Moloney LTR.  The
transformation efficiency was comparable to EC2, a
previously characterized mutation of human c-<u>raf</u>-1 cDNA
5    (G. Heidecker et al., <u>Mol. Cell. Biol.</u> 10, 2503 (1990);
C. Wasylyk et al., <u>Mol. Cell. Biol.</u> 9, 2247 (1989)) and
~20 fold lower than the v-<u>raf</u> oncogene.


                    *    *    *    *    *

10        All publications mentioned hereinabove are
hereby incorporated in their entirety by reference.


          While the foregoing invention has been
described in some detail for purposes of clarity and
15   understanding, it will be appreciated by one skilled in
the art from a reading of this disclosure that various
changes in form and detail can be made without departing
from the true scope of the invention and appended
claims.

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## SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: Rapp, Ulf R.
                Storm, Stephen M.

  (ii) TITLE OF INVENTION: DETECTION METHOD FOR C-RAF-1 GENES

 (iii) NUMBER OF SEQUENCES: 12

  (iv) CORRESPONDENCE ADDRESS:
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      (B) STREET: Suite 400, 133 Carnegie Way, N.W.
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      (D) STATE: Georgia
      (E) COUNTRY: USA
      (F) ZIP: 30303-1031

   (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

  (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER:
      (B) FILING DATE:
      (C) CLASSIFICATION:

 (viii) ATTORNEY/AGENT INFORMATION:
      (A) NAME: Perryman, David G.
      (B) REGISTRATION NUMBER: 33,438
      (C) REFERENCE/DOCKET NUMBER: 1414.0421

  (ix) TELECOMMUNICATION INFORMATION:
      (A) TELEPHONE: (404) 688-0770
      (B) TELEFAX: (404) 688-9880

(2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 648 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1          5              10             15

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```
Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
        20              25              30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35              40              45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50              55              60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65              70              75              80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
            115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165             170             175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
            195             200             205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210             215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
    275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290             295             300
```

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Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305               310           315                   320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325               330               335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
              340           345               350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
          355           360           365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
   370               375               380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385               390               395               400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
              405               410               415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
          420               425               430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
      435               440               445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450               455               460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465               470               475               480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
              485               490               495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
          500               505               510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
      515               520               525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
      530               535               540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545               550               555               560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
              565               570               575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
              580               585               590

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```
Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
    595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610             615             620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635             640

Thr Ser Pro Arg Leu Pro Val Phe
            645
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5               10              15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
        20              25              30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35              40              45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50              55              60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65              70              75              80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
            85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
            165             170             175
```

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Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
              180             185              190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
          195             200             205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210             215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
              245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
          260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
    275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290             295             300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
              325             330             335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
          340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
    355             360             365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
370             375             380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
              405             410             415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
          420             425             430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
    435             440             445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450             455             460

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Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485             490             495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
        500             505             510

Ala Pro Glu Val Val Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
        515             520             525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
        530             535             540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555             560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
            565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
        580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
        595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
        610             615             620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635             640

Thr Ser Pro Arg Leu Pro Val Phe
            645

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

    Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
    1           5           10              15

    Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20              25              30

    Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
            35              40              45

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Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50              55              60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65              70              75              80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
            85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
        130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
            165             170             175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195             200             205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
210             215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290             295             300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325             330             335

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Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
            355             360             365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
            370             375             380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405             410             415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420             425             430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
            435             440             445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
            450             455             460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485             490             495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500             505             510

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
            515             520             525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
            530             535             540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555             560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
            565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
            595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
            610             615             620

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```
Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635             640

Thr Ser Pro Arg Leu Pro Val Phe
            645
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5               10              15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
        20              25              30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35              40              45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50              55              60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65              70              75              80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
            85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
            165             170             175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195             200             205
```

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Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
210                 215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290             295             300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325             330             335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
    355             360             365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370             375             380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405             410             415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420             425             430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
    435             440             445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450             455             460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485             490             495

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```
Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500                 505             510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Ser Gln
            515             520             525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530             535             540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555             560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
                565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
            595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610             615             620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635             640

Thr Ser Pro Arg Leu Pro Val Phe
            645
```

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5               10              15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20              25              30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
    35              40              45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50              55              60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65              70              75              80
```

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Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                    85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165             170             175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195             200             205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210             215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290             295             300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325             330             335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355             360             365

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His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370             375             380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405             410             415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420             425             430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435             440             445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450             455             460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485             490             495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500             505             510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
    515             520             525

Ser Thr Cys Thr Phe Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530             535             540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555             560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
            565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
    595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610             615             620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635             640

Thr Ser Pro Arg Leu Pro Val Phe
            645

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(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AACTTGTGGT GGTTGGACCT                                                    20

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCATCCACAA AGTGATTCTG                                                    20

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGAGACCAA GTTTCAGATG                                                    20

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCGTGCAAGC ATTGATATCC                                                    20

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1947 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATGGAGCACA  TACAGGGAGC  TTGGAAGACG  ATCAGCAATG  GCTTTGGACT  CAAAGATGCG    60

GTGTTTGATG  GCTCCAGCTG  CATCTCCCCT  ACCATTGTTC  AGCAGTTTGG  CTATCAGCGC   120

CGGGCCTCAG  ATGATGGCAA  GCTCACGGAT  TCTTCTAAGA  CAAGCAATAC  TATCCGGGTT   180

TTCTTGCCGA  ATAAGCAAAG  GACTGTGGTC  AATGTGCGGA  ATGGAATGAG  CTTACATGAC   240

TGCCTTATGA  AAGCTCTGAA  GGTGAGAGGC  CTGCAGCCAG  AGTGCTGTGC  AGTGTTCAGA   300

CTTCTCCAGG  AACACAAAGG  TAAGAAAGCA  CGCTTAGATT  GGAACACCGA  TGCCGCCTCT   360

CTGATTGGAG  AAGAACTGCA  AGTGGATTTT  TTGGATCATG  TTCCCATCAC  AACTCACAAC   420

TTTGCTCGGA  AAACGTTCCT  GAAGCTTGCA  TTCTGTGACA  TCTGTCAGAA  GTTCCTGCTA   480

AATGGATTTC  GATGTCAGAC  TTGTGGCTAC  AAGTTTCATG  AGCACTGTAG  CACCAAAGTA   540

CCTACTATGT  GTGTGGACTG  GAGTAATATC  AGACAGCTCT  TGCTGTTTCC  AAATTCCACT   600

GTTGGTGACA  GTGGAGTCCC  AGCACCACCT  TCTTTCCCAA  TGCGTCGGAT  GCGAGAATCT   660

GTTTCCCGGA  TGCCTGCTAG  TTCCCAGCAC  AGATACTCTA  CACCCCATGC  CTTCACTTTC   720

AACACCTCCA  GCCCTTCCTC  AGAAGGTTCC  CTCTCCCAGA  GGCAGAGGTC  AACGTCCACT   780

CCCAATGTCC  ACATGGTCAG  CACCACCCTG  CATGTGGACA  GCAGGATGAT  TGAGGATGCA   840

ATTCGAAGTC  ACAGTGAATC  AGCCTCACCT  TCAGCCCTGT  CCAGCAGCCC  AAACAACCTG   900

GGTCCAACAG  GCTGGTCACA  GCCCAAAACC  CCCGTGCCAG  CACAAAGAGA  GCGGGCACCA   960

GGATCTGGGA  CCCAGCAAAA  AAACAAAATT  AGGCCTCGTG  GGCAGAGAGA  CTCGAGTTAT  1020

TACTGGGAAA  TAGAAGCCAG  TGAGGTGATG  CTGTCTACTC  GGATCGGGTC  AGGTTCCTTT  1080

GGCACTGTGT  ACAAGGGCAA  GTGGCATGGA  GATGTTGCAG  TAAAGATCCT  AAAGGTGGTT  1140

GACCCAACTC  CAGAGCAACT  TCAGGCCTTC  AGGAACGAGG  TGGCTGTTTT  GCGCAAAACA  1200

CGGCATGTTA  ACATCCTGCT  GTTCATGGGG  TACATGACAA  AGGACAACCT  GGCGATTGTG  1260

ACTCAGTGGT  GTGAAGGCAG  CAGTCTCTAC  AAACACCTGC  ATGTCCAGGA  GACCAAATTC  1320

CAGATGTTCC  AGCTAATTGA  CATTGCCCGA  CAGACAGCTC  AGGGAATGGA  CTATTTGCAT  1380

GCAAAGAACA  TCATCCACAG  AGACATGAAA  TCCAACAATA  TATTTCTCCA  TGAAGGCCTC  1440

ACGGTGAAAA  TTGGAGATTT  TGGTTTGGCA  ACAGTGAAGT  CACGCTGGAG  TGGTTCTCAG  1500

CAGGTTGAAC  AGCCCACTGG  CTCTGTGCTG  TGGATGGCCC  CAGAAGTAAT  CCGGATGCAG  1560
```

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GATGACAACC CGTTCAGCTT CCAGTCCGAC GTGTACTCGT ACGGCATCGT GCTGTACGAG 1620

CTGATGGCTG GGGAGCTTCC CTACGCCCAC ATCAACAACC GAGACCAGAT CATCTTCATG 1680

GTAGGCCGTG GGTATGCATC CCCTGATCTC AGCAGGCTCT ACAAGAACTG CCCCAAGGCA 1740

ATGAAGAGGT TGGTGGCTGA CTGTGTGAAG AAAGTCAAAG AAGAGAGACC TTTGTTTCCC 1800

CAGATCCTGT CTTCCATCGA GCTGCTTCAG CACTCTCTGC CGAAAATCAA CAGGAGCGCC 1860

TCTGAGCCTT CCCTGCATCG GGCAGCTCAC ACTGAGGACA TCAATGCTTG CACGCTGACT 1920

ACATCCCCAA GGCTACCAGT CTTCTAG .                                      1947


(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1947 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear


       (ix) FEATURE:
            (A) NAME/KEY: CDS
            (B) LOCATION: 1..1944


       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG GAG CAC ATA CAG GGA GCT TGG AAG ACG ATC AGC AAT GGT TTT GGA    48

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
 1               5                  10                  15


TTC AAA GAT GCC GTG TTT GAT GGC TCC AGC TGC ATC TCT CCT ACA ATA    96

Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
                20                  25                  30


GTT CAG CAG TTT GGC TAT CAG CGC CGG GCA TCA GAT GAT GGC AAA CTC   144

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
            35                  40                  45


ACA GAT CCT TCT AAG ACA AGC AAC ACT ATC CGT GTT TTC TTG CCG AAC   192

Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
        50                  55                  60


AAG CAA AGA ACA GTG GTC AAT GTG CGA AAT GGA ATG AGC TTG CAT GAC   240

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65                  70                  75                  80

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TGC CTT ATG AAA GCA CTC AAG GTG AGG GGC CTG CAA CCA GAG TGC TGT  288

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85              90                      95

GCA GTG TTC AGA CTT CTC CAC GAA CAC AAA GGT AAA AAA GCA CGC TTA  336

Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

GAT TGG AAT ACT GAT GCT GCG TCT TTG ATT GGA GAA GAA CTT CAA GTA  384

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
            115             120             125

GAT TTC CTG GAT CAT GTT CCC CTC ACA ACA CAC AAC TTT GCT CGG AAG  432

Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys
130             135             140

ACG TTC CTG AAG CTT GCC TTC TGT GAC ATC TGT CAG AAA TTC CTG CTC  480

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

AAT GGA TTT CGA TGT CAG ACT TGT GGC TAC AAA TTT CAT GAG CAC TGT  528

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
            165             170             175

AGC ACC AAA GTA CCT ACT ATG TGT GTG GAC TGG AGT AAC ATC AGA CAA  576

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

CTC TTA TTG TTT CCA AAT TCC ACT ATT GGT GAT AGT GGA GTC CCA GCA  624

Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
            195             200             205

CTA CCT TCT TTG ACT ATG CGT CGT ATG CGA GAG TCT GTT TCC AGG ATG  672

Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
210             215             220

CCT GTT AGT TCT CAG CAC AGA TAT TCT ACA CCT CAC GCC TTC ACC TTT  720

Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

AAC ACC TCC AGT CCC TCA TCT GAA GGT TCC CTC TCC CAG AGG CAG AGG  768

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245             250             255

TCG ACA TCC ACA CCT AAT GTC CAC ATG GTC AGC ACC ACG CTG CCT GTG  816

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```
Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
        260                 265             270

GAC AGC AGG ATG ATT GAG GAT GCA ATT CGA AGT CAC AGC GAA TCA GCC   864

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275                 280             285

TCA CCT TCA GCC CTG TCC AGT AGC CCC AAC AAT CTG AGC CCA ACA GGC   912

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
        290             295             300

TGG TCA CAG CCG AAA ACC CCC GTG CCA GCA CAA AGA GAG CGG GCA CCA   960

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

GTA TCT GGG ACC CAG GAG AAA AAC AAA ATT AGG CCT CGT GGA CAG AGA  1008

Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325             330             335

GAT TCA AGC TAT TAT TGG GAA ATA GAA GCC AGT GAA GTG ATG CTG TCC  1056

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340             345             350

ACT CGG ATT GGG TCA GGC TCT TTT GGA ACT GTT TAT AAG GGT AAA TGG  1104

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355             360             365

CAC GGA GAT GTT GCA GTA AAG ATC CTA AAG GTT GTC GAC CCA ACC CCA  1152

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
        370             375             380

GAG CAA TTC CAG GCC TTC AGG AAT GAG GTG GCT GTT CTG CGC AAA ACA  1200

Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

CGG CAT GTG AAC ATT CTG CTT TTC ATG GGG TAC ATG ACA AAG GAC AAC  1248

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405             410             415

CTG GCA ATT GTG ACC CAG TGG TGC GAG GGC AGC AGC CTC TAC AAA CAC  1296

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
        420             425             430

CTG CAT GTC CAG GAG ACC AAG TTT CAG ATG TTC CAG CTA ATT GAC ATT  1344
```

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Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435                 440                 445

GCC CGG CAG ACG GCT CAG GGA ATG GAC TAT TTG CAT GCA AAG AAC ATC 1392

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450                 455                 460

ATC CAT AGA GAC ATG AAA TCC AAC AAT ATA TTT CTC CAT GAA GGC TTA 1440

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465                 470                 475                 480

ACA GTG AAA ATT GGA GAT TTT GGT TTG GCA ACA GTA AAG TCA CGC TGG 1488

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485                 490                 495

AGT GGT TCT CAG CAG GTT GAA CAA CCT ACT GGC TCT GTC CTC TGG ATG 1536

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
        500                 505                 510

GCC CCA GAG GTG ATC CGA ATG CAG GAT AAC AAC CCA TTC AGT TTC CAG 1584

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
        515                 520                 525

TCG GAT GTC TAC TCC TAT GGC ATC GTA TTG TAT GAA CTG ATG ACG GGG 1632

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Thr Gly
        530                 535                 540

GAG CTT CCT TAT TCT CAC ATC AAC AAC CGA GAT CAG ATC ATC TTC ATG 1680

Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545                 550                 555                 560

GTG GGC CGA GGA TAT GCC TCC CCA GAT CTT AGT AAG CTA TAT AAG AAC 1728

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn
            565                 570                 575

TGC CCC AAA GCA ATG AAG AGG CTG GTA GCT GAC TGT GTG AAG AAA GTA 1776

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580                 585                 590

AAG GAA GAG AGG CCT CTT TTT CCC CAG ATC CTG TCT TCC ATT GAG CTG 1824

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
        595                 600                 605

CTC CAA CAC TCT CTA CCG AAG ATC AAC CGG AGC GCT TCC GAG CCA TCC 1872

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser

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```
        610                    615                    620
        TTG CAT CGG GCA GCC CAC ACT GAG GAT ATC AAT GCT TGC ACG CTG ACC 1920

        Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
        625                    630                635                    640

        ACG TCC CCG AGG CTG CCT GTC TTC TAG                                1947

        Thr Ser Pro Arg Leu Pro Val Phe
                        645
```

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
 1               5                  10                  15

Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20                  25                 30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35                  40                  45

Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50                      55                  60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65                  70                  75                      80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85                  90                      95

Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
            100                 105                 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
            115                 120                 125

Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys
        130                 135                 140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145                 150                 155                 160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165                 170                 175
```

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Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
          180               185               190

Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
          195               200               205

Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
          210               215               220

Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225               230               235               240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245               250               255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
                260               265               270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
          275               280               285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
          290               295               300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305               310               315               320

Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325               330               335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
          340               345               350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
          355               360               365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
          370               375               380

Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385               390               395               400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
                405               410               415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
                420               425               430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
          435               440               445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450               455               460

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```
Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465              470              475         His          480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
              485              490              495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
              500              505              510

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
              515              520              525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Thr Gly
              530              535              540

Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545              550              555              560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn
              565              570              575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
              580              585              590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
              595              600              605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
              610              615              620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625              630              635              640

Thr Ser Pro Arg Leu Pro Val Phe
              645
```

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WHAT IS CLAIMED IS:

1. A method of identifying an individual at an increased risk for developing cancer, comprising:

amplifying a region of the c-raf-1 gene of said individual;

analyzing products of said amplification for evidence of mutation; and

classifying an individual having one or more mutations in said region as having an increased risk for developing cancer.

2. The method according to claim 1, wherein said region encodes at least amino acids 514 to 535 of SEQ ID NO:12.

3. The method according to claim 2, wherein said region encodes at least amino acids 500 to 550 of SEQ ID NO:12.

4. The method according to claim 3, wherein said region encodes at least amino acids 450 to 630 of SEQ ID NO:12.

5. The method according to claim 1, wherein said products are analyzed by DNA sequencing.

6. The method according to claim 1, wherein said amplification is effected using a polymerase chain reaction (PCR).

7. The method according to claim 6, wherein said PCR employs a primer comprising SEQ ID NO:7 and a primer comprising SEQ ID NO:8.

8. A method for determining a prognosis in patients afflicted with cancer, comprising:

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    amplifying a region of the c-raf-1 gene of said
individual;

    analyzing products of said amplification for
evidence of mutation; and

    classifying a patient having no mutation in said
region as being less likely to suffer disease relapse or
having an increased chance of survival than a patient
having one or more mutations in said region.


    9. The method according to claim 8, wherein said
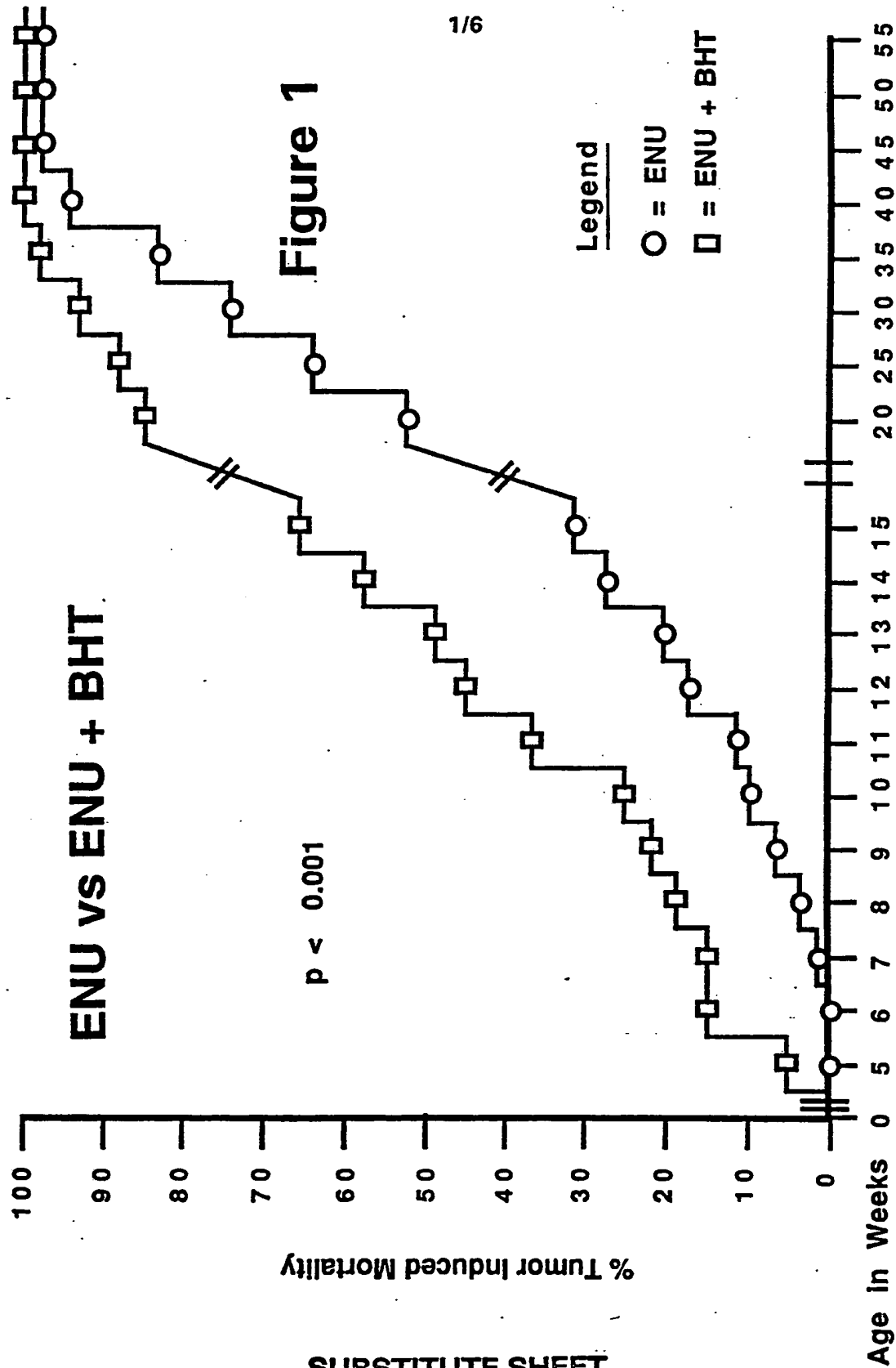region encodes at least amino acids 514 to 535 of SEQ ID
NO:12.


    10.   The method according to claim 9, wherein said
region encodes at least amino acids 500 to 550 of SEQ ID
NO:12.


    11.   The method according to claim 10, wherein said
region encodes at least amino acids 450 to 630 of SEQ ID
NO:12.


    12.   The method according to claim 9, wherein said
products are analyzed by DNA sequencing.


    13.   The method according to claim 9, wherein said
amplification is effected using polymerase chain
reaction (PCR).


    14.   The method according to claim 13, wherein said
PCR employs a primer comprising SEQ ID NO:7 and a primer
comprising SEQ ID NO:8.


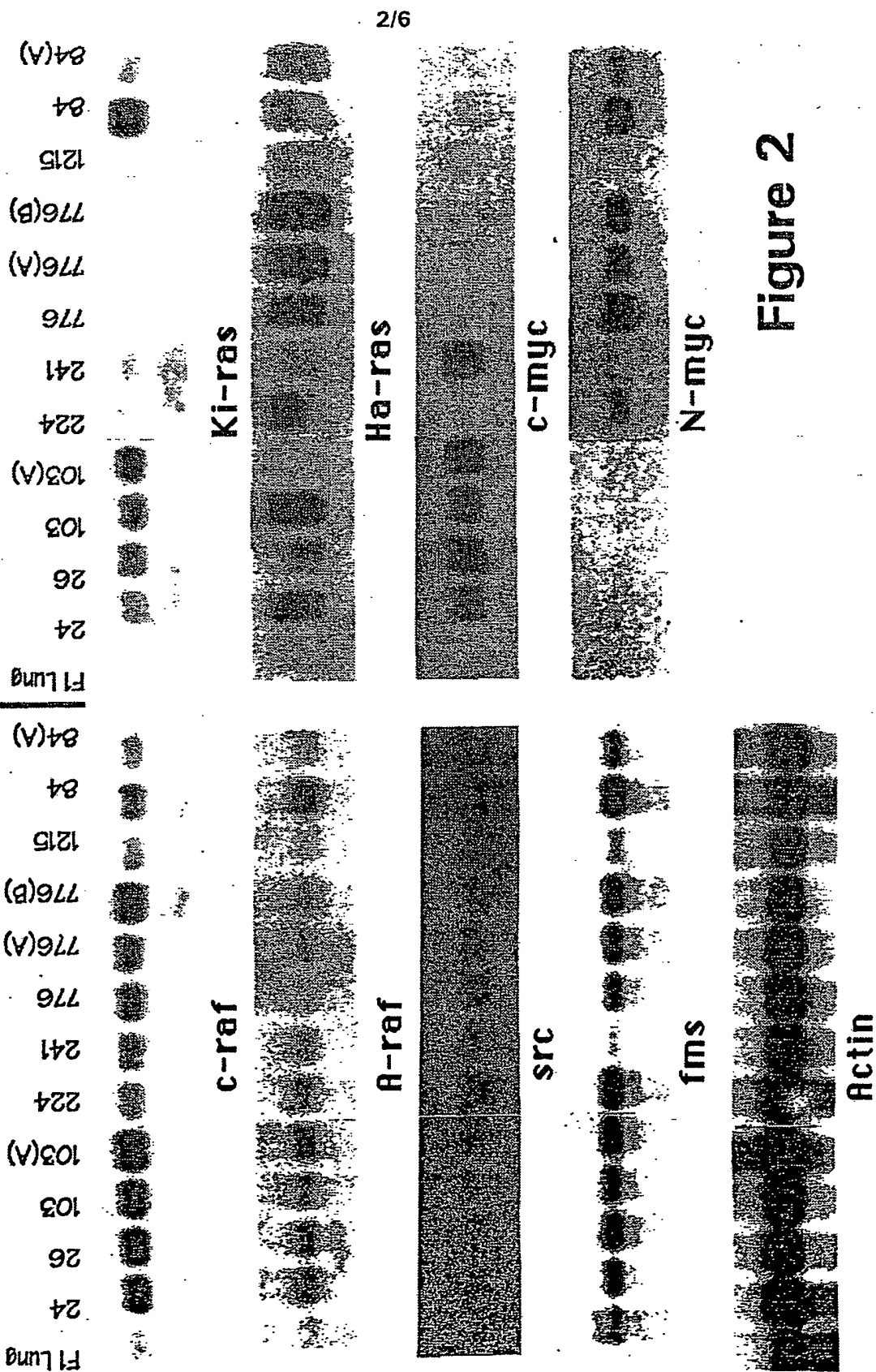    15.   A method for determining the proper course of
treatment for a patient afflicted with cancer,
comprising:

    amplifying a region of the c-raf-1 gene of said
patient;

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analyzing products of said amplification for
evidence of mutation;

identifying a patient having at least one mutation
in said region, which patient may require treatment
proper for a patient having a lesser chance of survival
or decreased time to relapse; and

identifying a patient lacking mutations in said
region, which patient may require treatment proper for a
patient having a greater chance of survival or being
less likely to suffer disease relapse.


16.    The method according to claim 15, wherein said
region encodes at least amino acids 514 to 535 of SEQ ID
NO:12.


17.    The method according to claim 16, wherein said
region encodes at least amino acids 500 to 550 of SEQ ID
NO:12.


18.    The method according to claim 17, wherein said
region encodes at least amino acids 450 to 630 of SEQ ID
NO:12.


19.    The method according to claim 16, wherein said
products are analyzed by DNA sequencing.


20.    The method according to claim 16, wherein said
amplification is effected using a polymerase chain
reaction (PCR).


21.    The method according to claim 20, wherein said
PCR employs a primer comprising SEQ ID NO:7 and a primer
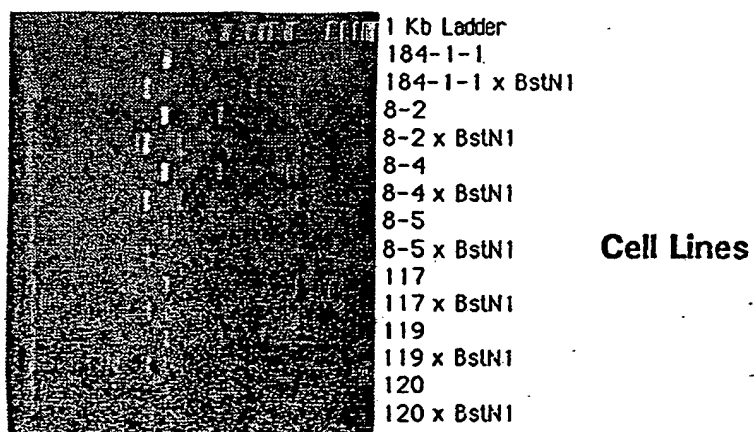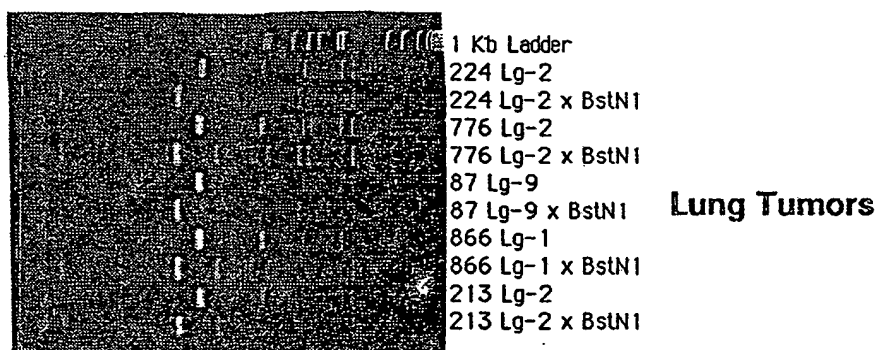comprising SEQ ID NO:8.

Figure 1

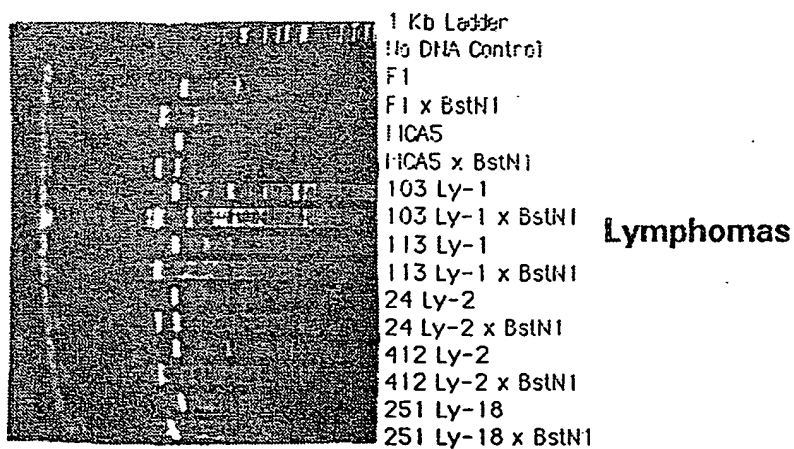ENU vs ENU + BHT

p < 0.001

Legend

O = ENU

□ = ENU + BHT

Figure 2

# Figure 3



| 1 Kb Ladder |
| No DNA Control |
| F1 |
| F1 x BstN1 |
| HCA5 |
| HCA5 x BstN1 |
| 103 Ly-1 |
| 103 Ly-1 x BstN1 |
| 113 Ly-1 |
| 113 Ly-1 x BstN1 |
| 24 Ly-2 |
| 24 Ly-2 x BstN1 |
| 412 Ly-2 |
| 412 Ly-2 x BstN1 |
| 251 Ly-18 |
| 251 Ly-18 x BstN1 |

**Lymphomas**



| 1 Kb Ladder |
| 224 Lg-2 |
| 224 Lg-2 x BstN1 |
| 776 Lg-2 |
| 776 Lg-2 x BstN1 |
| 87 Lg-9 |
| 87 Lg-9 x BstN1 |
| 866 Lg-1 |
| 866 Lg-1 x BstN1 |
| 213 Lg-2 |
| 213 Lg-2 x BstN1 |

**Lung Tumors**



| 1 Kb Ladder |
| 184-1-1 |
| 184-1-1 x BstN1 |
| 8-2 |
| 8-2 x BstN1 |
| 8-4 |
| 8-4 x BstN1 |
| 8-5 |
| 8-5 x BstN1 |
| 117 |
| 117 x BstN1 |
| 119 |
| 119 x BstN1 |
| 120 |
| 120 x BstN1 |

**Cell Lines**

4/6

# Figure 4



pBR322 + HaeIII

Probe

tRNA

v-raf

NFS/AKR F₁

24 LY-1

26 LG-1

103 LG-1

776 LG-2

145 LG-3

776 LG-3

776 LG-3A

**Figure 5**

Mouse Raf-1

Met 62    194   253 269   338        CR3        627 648

CR1        CR2

500    QVEQPTGSVLWMAPEVIRMQDDNPFSFQSDVYSYGIVLYELMAGELPYAH    550

Isoleucine to Valine

Aspartic Acid to Asparagine

Phenylalanine to Serine

TCTF

# Figure 6

## 1. Polymerase Chain Reaction (PCR) Amplification of Target DNA
## Either Genomic DNA or cDNA

Nucleotide 1307 of coding        Nucleotide 1326 of coding

Primer 1 = 5'- AGGAGACCAAGTTTCAGATG -3'

Nucleotide 1915 of coding        Nucleotide 1896 of coding

Primer 2 = 5'- GCGTGCAAGCATTGATATCC -3'

PCR Cycles:     94 $^{\circ}$ C, 1 minute

55 $^{\circ}$ C, 1 minute    Repeat 35 cycles

72 $^{\circ}$ C, 1 minute
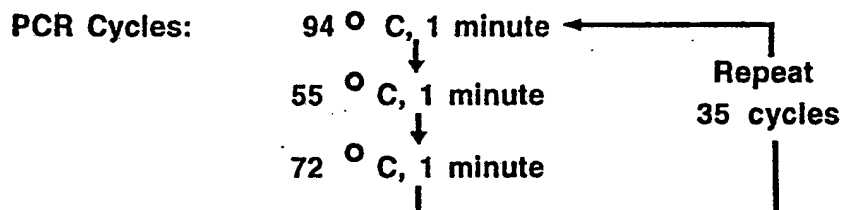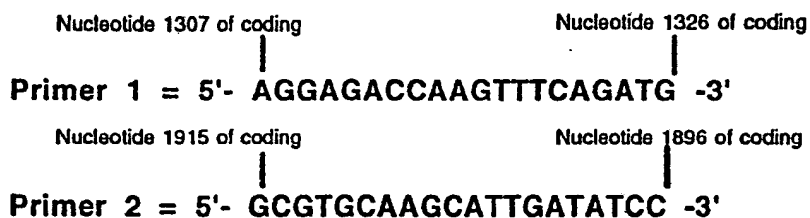
## 2. Isolate Product

## 3. Digestion with SphI and BglII

## 3. Direct Sequencing Asymmetric PCR

## 5. Standard Di-Deoxy Sequencing

## 4. Clone into c-raf-1 Containing Vector

## 4. Clone into Alternate Vector

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 92/07817

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

**II. FIELDS SEARCHED**

Minimum Documentation Searched⁷

| Classification System | Classification Symbols |
|---|---|
| IPC5 | C 12 Q |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched⁸

**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

| Category* | Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹² | Relevant to Claim No.¹³ |
|---|---|---|
| A | WO, A1, 9112343 (CETUS CORPORATION) 22 August 1991, see the whole document | 1-21 |
| A | WO, A1, 9105064 (USA, REPR. BY THE SECR.,D. OF HEALTH/HUMAN S.) 18 April 1991, see the whole document | 1-21 |
| A | WO, A1, 9009456 (VIKTOR BALAZS) 23 August 1990, see the whole document | 1-21 |
| A | Chemical Abstracts, volume 104, no. 19, 12 May 1986, (Columbus, Ohio, US), Bonner Tom I. et al.: "The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene", see page 141, abstract 162785c, & Nucleic Acids Res. 1986, 14( 2), 1009-1015 | 1-21 |

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 14th December 1992 | 12 01 93 |

| International Searching Authority | Signature of Authorized Officer |
|---|---|
| EUROPEAN PATENT OFFICE | Mikael G:son Bergstrand |

Form PCT/ISA/210 (second sheet) (January 1985)

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/07817

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**Box I    Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Claims 1-21
   Diagnostic methods, c.f. PCT Rule 39(iv). Nevertheless, a search
   has been made concerning the subject matter of the application
   (the analytical method).

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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**Box II    Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**          ☐ The additional search fees were accompanied by the applicant's protest.

                               ☐ No protest accompanied the payment of additional search fees.

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Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on                02/12/92
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A1- 9112343 | 22/08/91 | AU-D- | 7758991 | 03/09/91 |
| | | EP-A- | 0514501 | 25/11/92 |
| WO-A1- 9105064 | 18/04/91 | AU-D- | 6606190 | 28/04/91 |
| | | CA-A- | 2067114 | 03/04/91 |
| | | EP-A- | 0494968 | 22/07/92 |
| WO-A1- 9009456 | 23/08/90 | EP-A- | 0458831 | 04/12/91 |

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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